Positively charged residues influence the degree of SecA dependence in protein translocation across the *E. coli* inner membrane

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Abstract

The *sec* machinery catalyzes the translocation of nascent polypeptide chains across the inner membrane of *E. coli*, yet some inner membrane proteins depend only weakly or not at all on an intact *sec* function for membrane insertion even though they have stretches of chain protruding into the periplasmic space. Earlier work has demonstrated that the length of a periplasmic loop correlates with its degree of *sec*-dependence. We now show that the content of positively charged residues in a translocated loop also correlates with the degree of dependence on SecA function, suggesting that arginines and lysines may be inherently difficult to move across the membrane during *sec*-dependent translocation.

Key words: Membrane protein; Protein export; Leader peptidase; SecA

1. Introduction

Most periplasmic and many inner membrane *E. coli* proteins depend on the so-called *sec* machinery [1,2] for translocation across or insertion into the inner membrane. However, certain inner membrane proteins can insert efficiently even under conditions where the function of the *sec* machinery has been compromised. In at least two cases, the degree of *sec*-dependence has been shown to be related to the length of the loop that is translocated to the periplasmic side of the membrane. Thus, although membrane insertion of the phage M13 procoat protein normally does not depend on the Sec translocase, insertion becomes *sec*-dependent when the length of the periplasmic loop is increased from 20 to 118 residues [3]. An even more clear-cut demonstration of the relation between *sec*-dependence and length is provided by certain constructs based on the leader peptidase (Lep) enzyme, where *sec*-dependence was found to increase linearly with the length of the translocated loop to reach a plateau at a length of ~60 residues [4]. A possible interpretation is that short loops require only one or a few rounds of ATP hydrolysis by the *sec* machinery [5,6], and thus that their translocation is relatively insensitive to treatments that interfere with the ATPase activity of the translocase.

These results do not rule out the possibility that other characteristics of a translocated loop may also influence its degree of *sec*-dependence. Since short (and hence probably *sec*-independent) but not long periplasmic loops in inner membrane proteins contain only few positively charged residues [7,8], there is a clear possibility that the number of arginines and lysines in a loop may also contribute to *sec*-dependence. By analyzing two different sets of Lep mutants we show that this indeed seems to be the case: the translocation of loops with a higher number of positively charged residues is more affected when the function of the SecA protein is blocked.

2. Materials and methods

2.1. Enzymes and chemicals

Trypsin, soybean trypsin inhibitor, chicken egg white lysozyme, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. All other enzymes were from Promega and Pharmacia (T7 DNA polymerase).

2.2. Strains and plasmids

Leader peptidase mutants were expressed from the pING1 plasmid [9] in *E. coli* strain MC1061 (ΔlacX74, araD139, Δ(ara, leu)7697, galU, galK, hsr, hsm, strA).

2.3. DNA techniques

Site-specific mutagenesis was performed according to the method of Kunkel [10], as modified by Geisselsoder et al. [11]. All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase. Cloning into the pING1 plasmid was performed as described [12].

2.4. Assay of membrane topology

E. coli strains transformed with the pING1 vector carrying mutant leader peptidase (*lep*) genes under the control of the arabinose promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, and all amino acids (50 μg/ml each) except methionine. Overnight cultures were diluted 1:25 in 1 ml fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2%) for 5 min, and labeled with [³⁵S]methionine (150 μCi/ml). After 1 min, non-radioactive methionine was added (500 μg/ml) and incubation was stopped by chilling on ice. Cells were spun at 15,000 rpm for 2 min, resuspended in ice-cold buffer (40% w/v sucrose, 33 mM Tris, pH 8.0), and incubated with lysozyme (5 μg/ml) and 1 mM EDTA for 15 min on ice. Aliquots of the cell suspension were incubated for 1 h on ice, either with 0.75 mg/ml TPCK-trypsin or with 0.75 mg/ml TPCK-trypsin + 1.0 mg/ml trypsin inhibitor + 0.33 mg/ml PMSF. After addition of trypsin inhibitor and PMSF, samples were acid-precipitated (trichlo-

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roacetic acid, 10% final conc.), resuspended in 10 mM Tris, 2% SDS, immunoprecipitated with antisera to Lep, OmpA (an outer membrane control, not shown), and AraB (a cytoplasmic control, not shown), washed, and analyzed by SDS-PAGE and fluorography.

The degree of SecA-dependence for the constructs shown in Fig. 1 was determined as follows: synthesis was induced with arabinose as above, and sodium azide was added (2 mM final concentration) 4 min after induction. 1 min later, [^{35}S]Met was added, and after an additional 1-min incubation cells were put on ice, converted to spheroplasts, trypsinized, immunoprecipitated, and analyzed by SDS-PAGE as above. In parallel samples, azide treatment was omitted. For both the azide-treated and non-treated samples, the fraction of counts in Fig. 1B,C, band b (trypsin-treated cells) relative to bands a + b + c, was calculated from:

$$f = \frac{7C_b/6}{C_a + 7C_b/6 + 7C_c/3}$$

(where C$_i$ is the background-corrected counts in band i in the trypsin-treated cells, and the factors 7/6 and 7/3 correct for the different numbers of methionines in the different molecules; note that band c is not shown in Fig. 1B,C but is indicated by * for mutant 4K5(88) in Fig.2B. The degree of sec-dependence was defined as:

$1-(f^{+az}/f^{-az})$.

The fractions of molecules with wild-type and 'inverted' topology in Fig. 2B,C were calculated from the bands marked b and *. Band b represents inverted molecules that have been cleaved by trypsin in the periplasmic P1 loop, while band * represents a trypsin-resistant frag-

ment produced by trypsin cleavage around residues 198–199 in the P2 domain. The fraction of inverted molecules is thus given by:

$$f = \frac{3C_b/6}{C_* + 3C_b/6}$$

where C$_i$ is the background-corrected counts in band i in the trypsin-treated cells, and the factor 3/6 corrects for the difference in the number of methionines between the two fragments.

3. Results

3.1. SecA-dependence correlates with the number of positively charged residues in a translocated loop

To study the possible influence of positively charged residues on sec-dependence, we chose a previously characterized 'inverted' leader peptidase construct with a periplasmic loop of 88 residues (Fig. 1A). This construct (mutant 4K5(88) [4]) has 9 positively charged residues in the periplasmic P1 loop, and its translocation is almost completely blocked in cells where the SecA ATPase activity is inhibited by treatment with 2 mM sodium azide [13]. The degree to which sodium azide blocked translo-
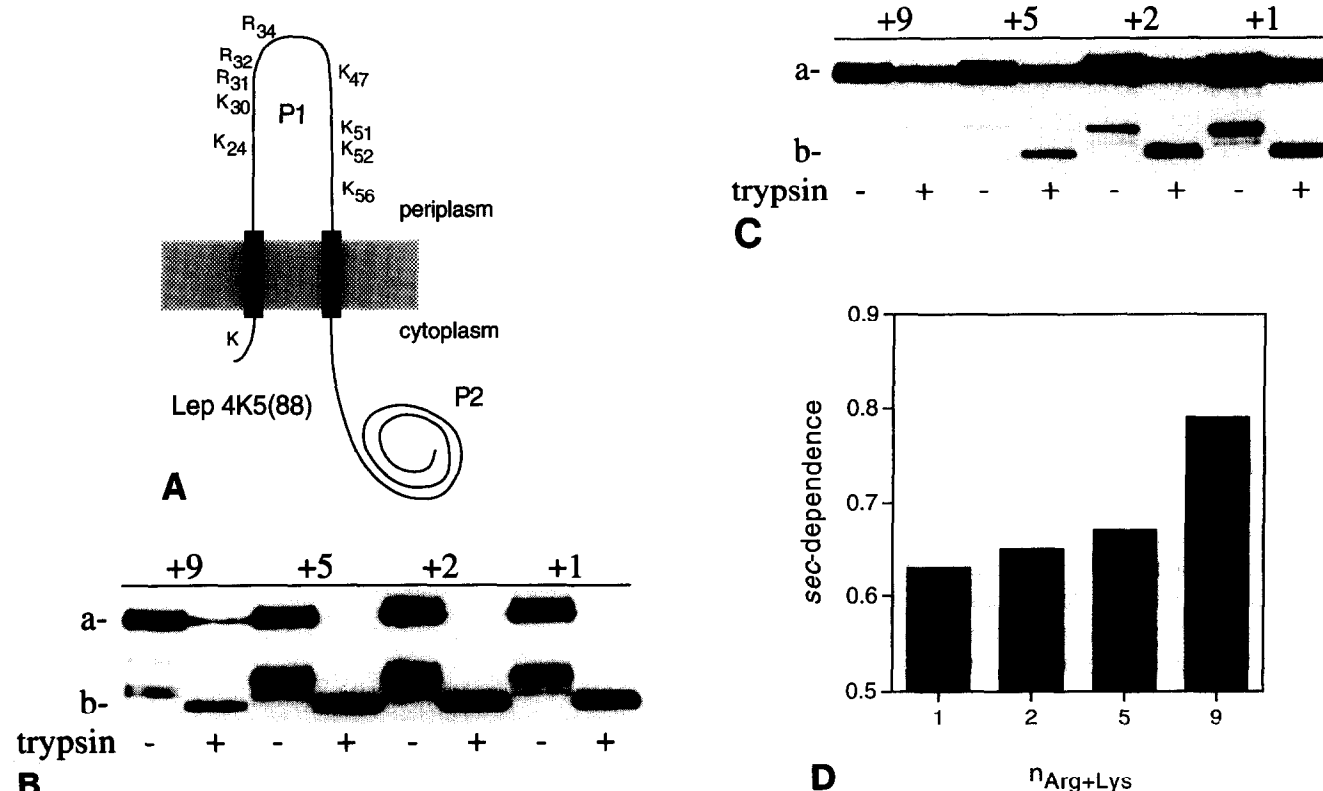

Fig. 1. SecA-dependence is a function of the number of positively charged residues in the translocated loop. (A) Topology of mutant 4K5(88) in the inner membrane. Note the location of the block of 4 closely spaced positively charged residues (positions 30–34 in the wild-type Lep sequence). This block was replaced by five Asn residues to make mutant 4K5(88)/ + 5. In mutant 4K5(88)/ + 2, lysines 51, 52, and 56 were also replaced by Asn. Finally, mutant 4K5(88)/ + 1 was made by replacing Lys[24] with Asn, leaving Lys[47] as the only positively charged residue in P1 (we have found that the codon for Arg[42] in the published lep sequence is incorrect and should read GCCGGG, coding for Ala-Gly). (B) Trypsinization of mutants 4K5(88)/ + n (n = 9, 5, 2, 1). Synthesis was induced by addition of arabinose, cells were pulse-labeled for 1 min with [^{35}S]methionine, converted to spheroplasts, incubated with trypsin, and immunoprecipitated with Lep antiserum. Band a is the full-length protein, band b is the trypsin-resistant fragment resulting from cleavage in the P1 loop. (C) Same as panel B, but pulse-labeled in the presence of 2 mM sodium azide to block the SecA ATPase. (D) Degree of SecA-dependence of the translocation of the P1 loop in mutants 4K5(88)/ + n (see section 2).

cation was progressively reduced, however, as the number of positively charged residues in the loop was reduced by replacing them with Asn residues (Fig. 1B–D). We conclude that, for constant length, the degree of dependence on SecA function can be affected by the number of positively charged residues in the loop.

3.2. 'Inversion' of protein topology: relationship between loop length and charge content

We have previously shown that, for a series of constructs similar to the 4K5(88) mutant but with loop lengths ranging from 39 to 88 residues, an increasing fraction of the molecules insert in an 'inverted' orientation as the loop length is increased [4] (Fig. 2A). Our interpretation was based on the idea that the N-terminal tail and the P1 loop compete for translocation across the membrane: while the 39 residue long P1 loop is too short

to be translocated by the sec machinery and too highly charged to be translocated by a sec-independent mechanism, the longer P1 loops become increasingly better substrates for the sec machinery and can hence compete more efficiently with the sec-independent N-terminal tail [14], leading to an increase in the fraction of molecules that insert with an inverted orientation.

According to this model, and knowing that positively charged residues seem to influence the degree of sec-dependence, we reasoned that the same set of loops, but now containing 5 rather than 9 positively charged residues, might shift to the inverted topology at shorter loop lengths. This expectation was indeed borne out (Fig. 2B,C): in the less charged series of mutants, a given fraction of inverted molecules was reached at about a 20 residue shorter loop length than for the highly charged constructs (Fig. 2D).
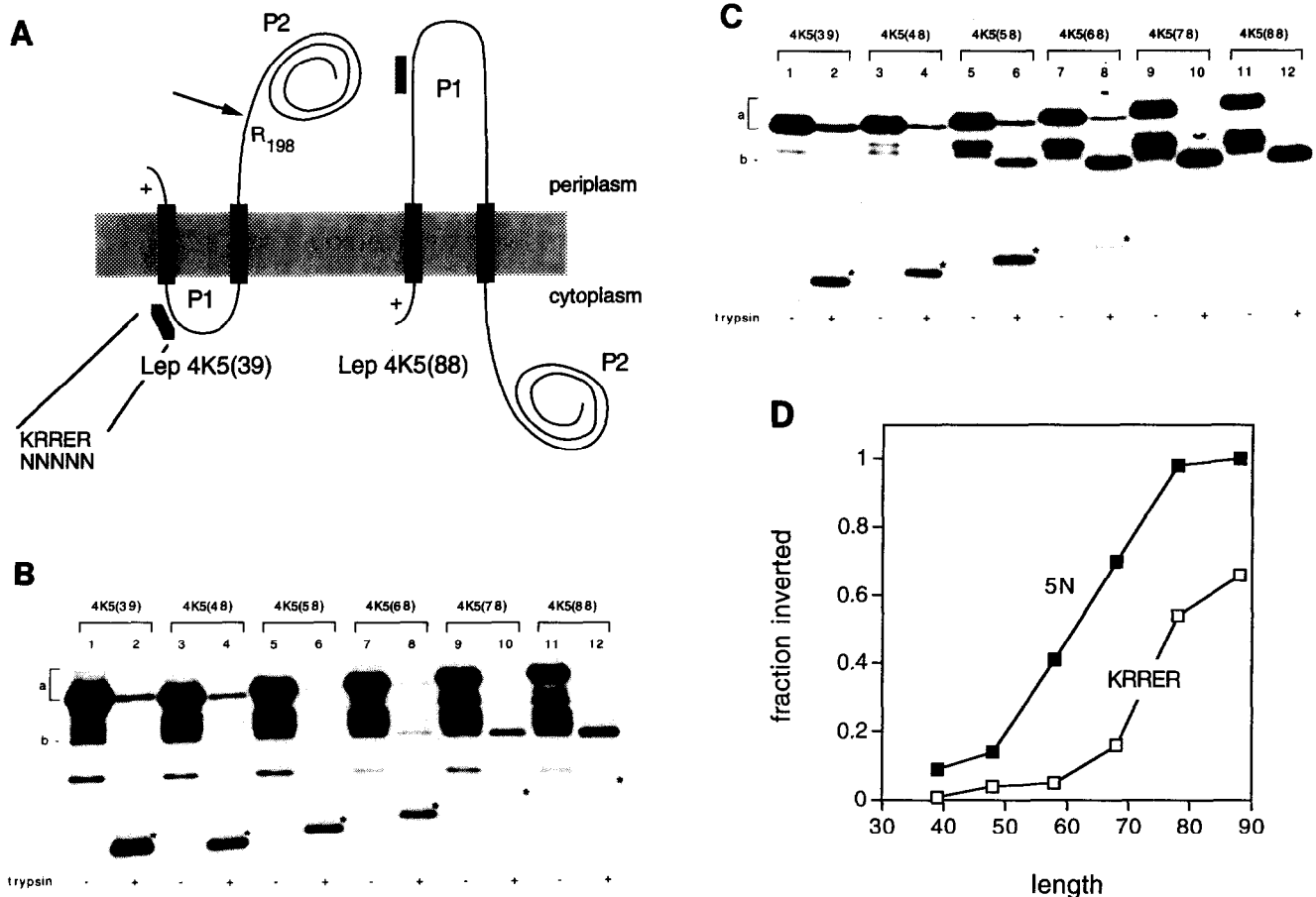

Fig. 2. 'Inversion' of protein topology: positively charged residues oppose translocation. (A) Topology of mutants 4K5(39) and 4K5(88) in the inner membrane. Note that the N-terminal tail and the P1 loop compete for translocation. The approximate location of the trypsin-sensitive site in the P2 domain that gives rise to the fragments marked * in panels B and C is indicated. (B) Trypsinization of mutants 4K5(l) (l = 39, 48, 58, 68, 78, 88). Synthesis was induced by addition of arabinose, cells were pulse-labeled for 1 min with [^{35}S]methionine, chased for 5 min with excess non-radioactive methionine, converted to spheroplasts, incubated with trypsin, and immunoprecipitated with Lep antiserum. Band a is the full-length protein, band b is the trypsin-resistant fragment resulting from cleavage of inverted molecules in the P1 loop, and the band marked * is the trypsin-resistant fragment resulting from cleavage of wild-type-orientated molecules in the P2 domain. As expected, the size of the latter increases with the size of the P1 loop, while the b fragment has a constant size. The gel has been over-exposed to bring out the bands marked * in the '+trypsin' lanes representing molecules cleaved in the P2 domain; as a result, a set of endogenous partial degradation products in the '−trypsin' lanes tend to blur these lanes. The full-length form (band a) is the dominating one for all constructs, however. (C) Same as panel B, but for the series of mutants where residues 30–34 (KRRER) have been changed to five consecutive Asn residues. (D) Fraction of inverted molecules calculated from the data in panels B and C (see section 2).

4. Discussion

In this communication, we have demonstrated that the degree of SecA-dependence, i.e. the degree to which translocation of a periplasmic loop is affected by azide-inhibition of the SecA ATPase activity, is a function not only of the overall size of the loop but also of its amino acid composition, notably its content of positively charged residues. As shown by the experiments based on the 4K5(88) mutant (Fig. 1), a clear reduction in SecA dependence is seen when the number of positively charged residues in the periplasmic loop is reduced from 9 to 1. The major drop in *sec*-dependence occurs when a block of four closely spaced positively charged residues (residues 30–34 in the wild-type sequence) is replaced by five uncharged asparagines; this same block of residues has previously been shown to function as a 'transloca-tion poison sequence' in the wild-type protein [15,16], and may thus be particularly difficult to translocate and particularly sensitive to inhibition of SecA function.

In the experiments reported in Fig. 2, the situation is a little more complex: here, the short N-terminal tail and the P1 loop compete for translocation [4]. When the poison sequence is replaced by uncharged residues, the inverted orientation becomes the preferred one at shorter overall loop lengths. This suggests that the P1 loop becomes more efficient in competing with the *sec*-independent N-terminal tail when the number of positively charged residues (or long blocks of such residues) is reduced, possibly because translocation of the P1 loop is less dependent on SecA function in this case.

One interpretation of our results is that the measured degree of SecA-dependence is related to the number of rounds of SecA-catalyzed ATP hydrolysis needed. If this is true, it would mean that positively charged residues are inherently difficult to translocate through the *sec* machinery and thus require a larger ATP expenditure. This

is reminiscent of the fully *sec*-independent translocation mechanism, for which we have recently shown that the electrochemical potential across the inner membrane opposes the translocation of positively charged residues [17].

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